

# Walsby's Square Bacterium: Fine Structure of an Orthogonal Procaryote

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The "square" bacterium, first described by Walsby from brine collected at the Red Sea shore [A. E. Walsby, *Nature* (London) **283**:69-71, 1980] was examined by electron microscopy. The cells appeared as flat rectangular boxes in scanning electron micrographs. In sections and freeze-fracture preparations, the edges looked more rounded. The thickness apparently remains constant as the cells grow and divide. Their sides were a few micrometers long, but the cells were only 0.25  $\mu\text{m}$  thick. They showed typical procaryote structure, with a regular cell wall and a gas vacuole fine structure similar to that of other halophilic procaryotes. The inner fracture faces of the cell membrane showed a much denser population of intramembrane particles than the outer fracture faces, but no patches of purple membrane, despite the presence of bacteriorhodospin-like pigment in the cell suspension. Morphologically identical cells have been found in brine from Baja California, Mexico.

A new morphological type of procaryote has recently been described by A. E. Walsby (8). The organism was found in a natural salt pond of a sabkha bordering the western shore, Gulf of Eilat, between Nabq and Ophira and near the southern tip, Sinai peninsula. The site consists of a roughly circular shallow depression about 500 m in diameter which is separated from the sea by low sand dunes. Seawater seeps through the sand and collects in the depression, where it is concentrated by evaporation. We visited the site in August 1980, when most of the depression was dry and only a crescent-shaped shallow pool remained at the northern end. It was 10 to 15 m wide, 150 m long, and not more than 20 to 50 cm deep. Salt had crystallized in a thick crust around the pool. Of 25 samples collected, 23 contained the organism described by Walsby in addition to other, mainly rod-shaped bacteria and some unicellular algae with the morphological characteristics of *Dunaliella* sp.

The "square" cells are flat rectangular boxes with perfectly straight edges measuring a few micrometers on the side, and their height is near the limit of resolution of a light microscope. The smallest cells are always square, measuring 2 by 2  $\mu\text{m}$ , whereas larger cells are often rectangular. They contain many gas vacuoles and also small granules which appear dark in the phase-contrast image. Our observations essentially agree with Walsby's description. He did not mention the dark granules, but a few can be seen in his pictures. They appear to be considerably more

abundant in our samples.

Halophilic bacteria have recently attracted increased interest mainly for two reasons. The extreme halophiles belong to a group of procaryotes so different from other bacteria that they have been classified as a new kingdom, the archaeobacteria (9), and some of them contain a photosynthetic system not based on chlorophyll, but on bacteriorhodopsin, a chromoprotein resembling the visual pigments of animals (6). Since the new organism discovered by Walsby is obviously halophilic, we decided to study it more closely to determine whether it would show these interesting features. We observed cells with an electron microscope rather than a light microscope to characterize them better morphologically. Attempts to grow the organism in the laboratory are under way.

## MATERIALS AND METHODS

Cells were sedimented by centrifugation of 6 liters of brine. This yielded approximately 20 ml of cell suspension (ca.  $10^9$  cells per ml) in which the square bacterium was the dominant morphological species. The cells were fixed by the addition of a 40% formaldehyde solution to a final concentration of 4% within 3 days after collection. The cells remained in this solution, and no visible change in their morphology was seen. All further processing was carried out 3 weeks later in San Francisco.

The cells were prepared for sectioning and freeze-fracture by standard techniques. No cryoprotectant was used, and the freezing medium was Freon 22 (5). Before embedding and sectioning, cells were postfixed

in  $\text{OsO}_4$  or in  $\text{KMnO}_4$  solution as described by Stoeckienus and Rowen (7); no significant difference was noted in the effects of the two fixatives. For scanning electron microscopy (SEM), the cells were allowed to settle on a polylysine-coated cover slip, exposed to  $\text{OsO}_4$  vapor for 2 h, washed with distilled water, dehydrated in ethanol, and after critical point drying, coated with gold. Preparations were viewed in a Siemens 101 or a Cambridge S 150 electron microscope.

## RESULTS

The concentrated cell suspension obtained by centrifugation had the reddish color characteristic of extreme halophiles and gave the typical carotenoid absorption spectrum. Flash spectroscopy showed a transient absorbance change indicative of the presence of bacteriorhodopsin (3) (Fig. 1). Light microscopy after centrifugation showed that the gas vacuoles had collapsed, as expected, and the dark granules were now much more prominent; no change in the shapes of the cells was detected (Fig. 2). Larger cells and especially the frequently found sheets of cells were often bent so that only part of the cell was in focus when a high-power objective lens was used and the plane of the cell was at a right angle to the viewing direction. Scanning electron microscopy confirmed the overall shapes of the cells and the fact that they were the dominant mor-

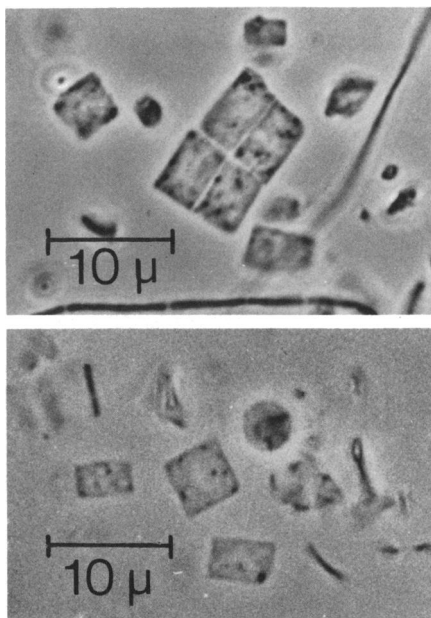


FIG. 2. Phase-contrast micrographs of cells after concentration. The gas vacuoles were collapsed by the centrifugation and are not visible. The dark granules are prominent. In the lower micrograph, two of the square cells are seen in profile in the upper left and lower right quadrant. Magnification,  $\times 1,600$ .

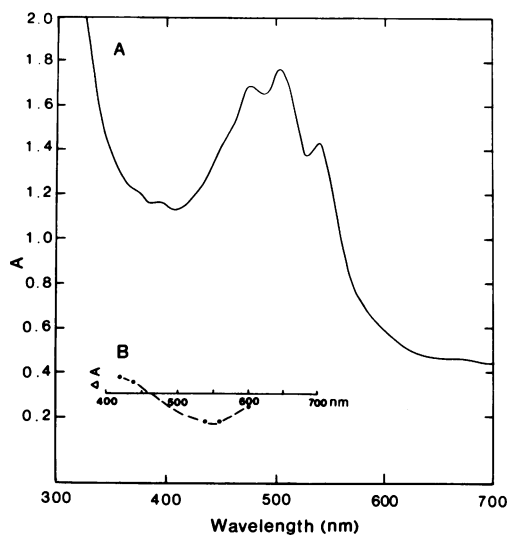


FIG. 1. Absorption (A) spectrum of cells concentrated from Red Sea brine. The absorption maxima at 540, 505, and 475 nm and the shoulder at 450 nm are characteristic for carotenoids found in extremely halophilic bacteria. Insert, Difference spectrum for the transient absorbance change 0.5 ms after an actinic flash at 500 nm. The minimum near 560 nm and maximum near 400 nm indicate the presence of bacteriorhodopsin-like pigment.

phological species (Fig. 3-5). It showed the cells to be uniformly  $0.25 \mu\text{m}$  thick and to have amazingly acute edges and corners. Bending around an axis parallel to the plane of the cells was even more pronounced than expected from light microscopy and may have been enhanced by the preparation technique (Fig. 3). The cells usually showed a rather smooth surface. Where structure was visible, it was irregular. There was little difference from the background (Fig. 4). The sheets of four or more adherent cells often seen in the light microscope were not observed in the electron microscope. They apparently had not withstood the preparation procedure. The size of the smallest cells was approximately  $2.0$  by  $2.0$  by  $0.25 \mu\text{m}$ , but squares twice that size and rectangles with dimensions of  $4.0$  by  $2.0 \mu\text{m}$  with no cell wall septa were often seen (Fig. 5). The thickness of the cells apparently remained constant. This suggests that the cells grow from smaller into larger squares of the same thickness and then undergo two divisions in rapid succession, with or without separation of the daughter cells after the first division. The larger sheets of squares seen in the light microscope may be explained by assuming that the cells fail to separate after the second division and then grow and divide synchronously, or that division is

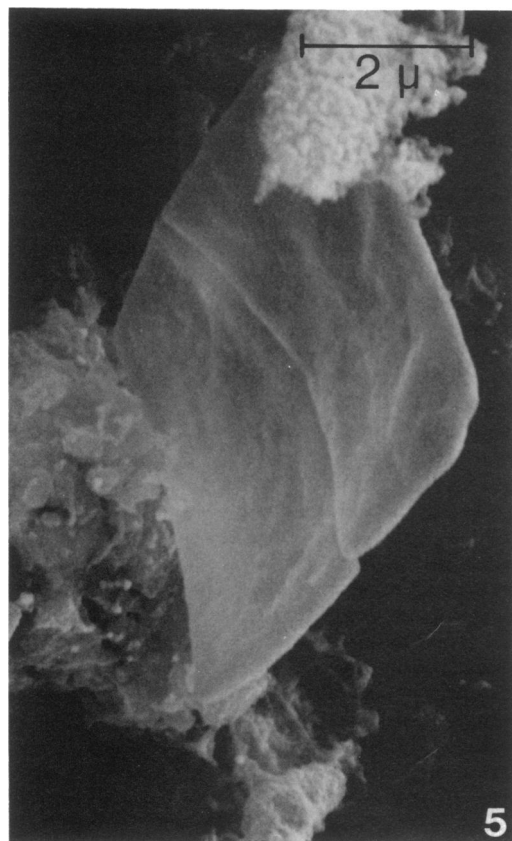
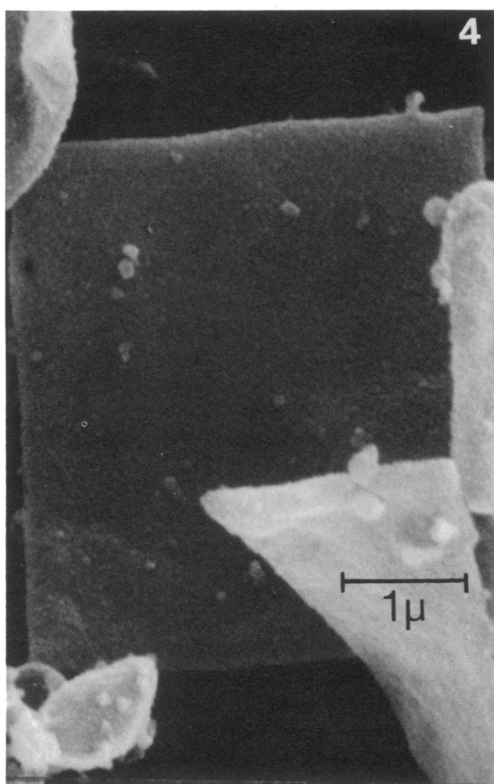
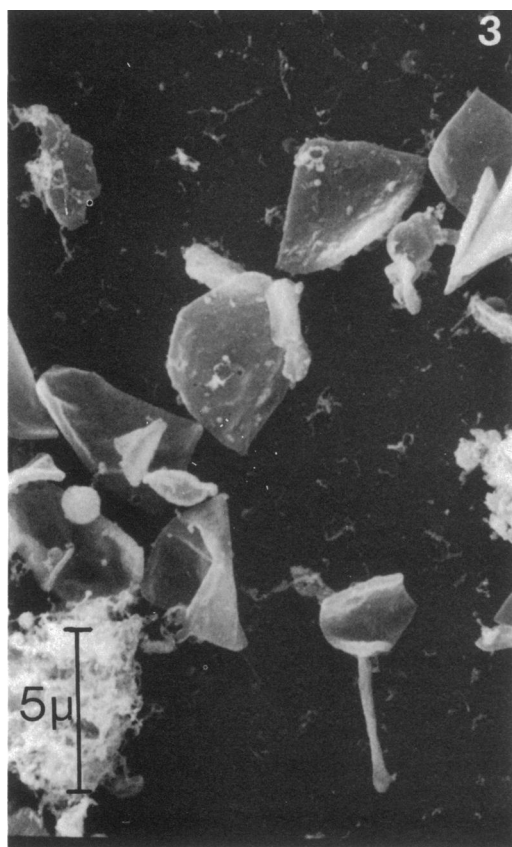


FIG. 3. Scanning electron micrograph of cell concentrate. Typical field at low magnification. Note the flat appearance of the edges of cells where they are turned up and are seen face on. Magnification,  $\times 4,500$ .

FIG. 4. Same as Fig. 3. One cell, approximately twice the size of the smallest cell, is lying flat on the substrate. Several other cells curve upwards and appear brighter because they are electrically charged by the scanning beam. Magnification,  $\times 18,000$ .

FIG. 5. A cell of the same size as that in Fig. 4, apparently shortly after the first division. A second division at a right angle to the first would generate four cells of the smallest size found. This is one of a pair of stereomicrographs, which show that the lower right hand edge of the cell pair curves upwards towards the viewer. Magnification,  $\times 12,000$ .

delayed until a higher multiple of the standard cell size is reached. We have, however, failed to find cells larger than 5 by 5  $\mu\text{m}$  in the scanning electron microscope, and cell division lines may not always be visible in the light microscope.

In the sectioned material, a given cell profile was not always easy to identify. Sections in the plane of the square cells were necessarily rare, and if the cells were bent, they could not encompass a whole cell. Fortunately, the square cells constituted the majority of the material, and in cross-section, they were easily recognized because they were thinner than any of the other cells present. The long, slender profiles which dominated in the sections must, therefore, belong to the square cells (Fig. 6 and 8). A rare, nearly complete in-plane section is shown in Fig. 7. The straight edges and sharp corners are obvious. The cytoplasm contains granules and fine strands of material, possibly ribosomes, and DNA similar to what is typically seen in other procaryotes, but rather less densely packed. The dense granules seen in the light microscope have apparently been dissolved and appear as oval empty spaces up to 0.3 or 0.4  $\mu\text{m}$  in diameter. In cross-sections, their longer axis can be seen to lie in the plane of the cell. The collapsed gas vacuoles are also easily recognized because they resemble unit membranes in cross-section but with free ends (5, 7). Sections normal to the plane of the cells show their width to be less uniform and their ends to be more rounded than one would expect from the scanning electron microscopy images. We ascribe this to irregular shrinkage during embedding because the thickness of the cells was often considerably less than the rather constant 0.25  $\mu\text{m}$  seen in the scanning micrographs, and the thinner the cells, the denser appeared the cytoplasm. The cells showed a thick wall over the plasma membrane, which appeared as a 3.0- to 4.0-nm-wide light line delimiting the cytoplasm (Fig. 9A). The appearance of the wall varied considerably. It often showed an inner, dense line and a broader band of lighter material on the outside or vice versa. The width varied considerably from approximately 15 to approximately 25 nm in what appeared to be cross-sections. It clearly had a regular in-plane structure. Prominent periodicities of 20 nm were often seen in slightly oblique sections, and more oblique cuts gave rise to complex patterns (Fig. 9B). We made no attempts to analyze the patterns, because we suspected that the population of square cells consisted of more than one species and because all cells did not have the same wall structure.

Freeze-fracture preparations confirmed the general shape of the cells and the presence of a

cell wall, cytoplasmic granules, and gas vacuoles. The varying thickness and rounded edges seen in cross fractures were similar to images seen in sections and again suggested that some deformation had occurred. The granules were either broken out of the fracture face, leaving round or oval depressions, or were drawn out into prominent, often bent, cone-shaped structures protruding from the fracture face (Fig. 10). Similar deformations have been shown to occur during freeze-fracture of latex spheres and a variety of biological structures, especially poly- $\beta$ -hydroxybutyrate granules in procaryotes (2, 4). In well-resolved replicas, the collapsed gas vacuoles show the typical cross striation with a 2.0-nm periodicity (5). In-plane fractures of the cell membrane showed a dense population of particles on the cytoplasmic leaflet and a much sparser particle distribution on the external leaflet, as do most cell membranes (Fig. 11). Even though flash spectroscopy indicated that bacteriorhodopsin was present, we only very rarely found small, ordered arrays of intramembrane particles, and they did not show the typical appearance of the purple membrane patches seen in other halobacteria (1). In slightly etched preparations, the cell wall was clearly visible and appeared approximately 15 nm wide. Its regular structure was clearly seen in face view and consisted of round particles with a spacing of approximately 23 nm (Fig. 11). The pattern appeared to be orthogonal or hexagonal, but etching in the high salt concentration required is difficult to obtain. We suspect that the cell population is not homogeneous in morphology, but we have not seen sufficient extended face views of the cell walls for an analysis. Fixation with  $\text{OsO}_4$  and subsequent washing with water did not preserve the structure in heavy metal-shadowed preparations.

## DISCUSSION

If there were any lingering doubts left as to whether or not the objects described by Walsby were indeed bacterial cells, the fine structure observations reported here should dispel them. Moreover, we have been able to grow the cells in enrichment culture by adding peptone medium to the natural brine, but growth of pure colonies on agar has not been achieved so far. Their size, their lack of a nuclear membrane, their wall structure, and the occurrence of gas vacuoles clearly identify them as procaryotes. They are obviously halophilic; whether or not they are extreme halophiles remains to be seen. Their apparently slow growth, red pigmentation, and regular cell wall structure are compatible with such a classification (5-7), but certainly not

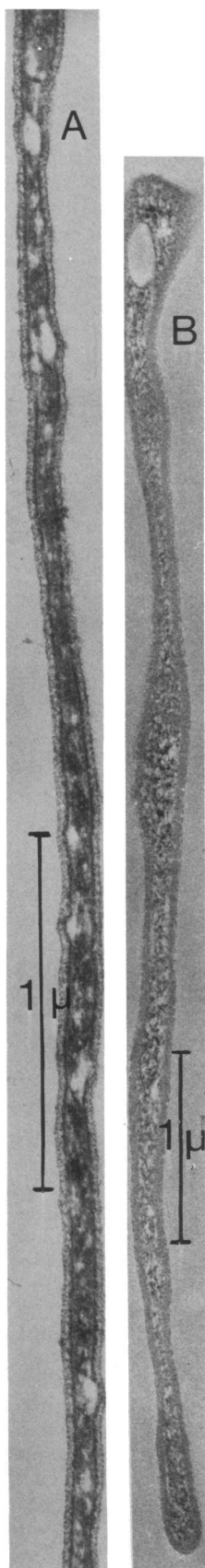


FIG. 6. Transmission electron micrographs of cross-sectioned cells. The cell shown in 6a is much narrower (approx.  $0.1\ \mu\text{m}$ ) and has a much denser cytoplasm than the cell in 6b. (Note difference in magnification.) The upper edge of the cell in 6b appears squared off but the lower edge is rounded. Compared to the appearance in scanning electron micrographs, the cells appear more irregular in shape. Magnifications,  $\times 54,000$  (6a) and  $\times 28,600$  (6b).

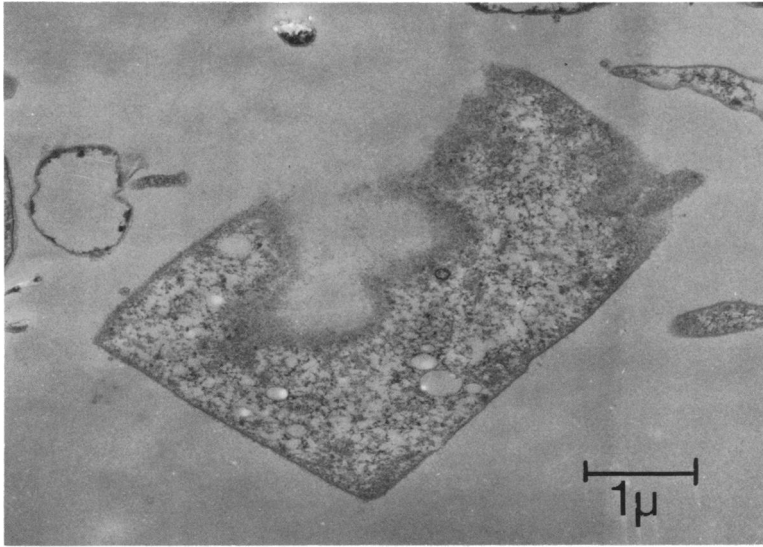


FIG. 7. A nearly in-plane section of the cells shows the straight edges and sharp corners. Empty oval spaces correspond to dark granules seen in phase-contrast pictures. Magnification,  $\times 14,300$ .

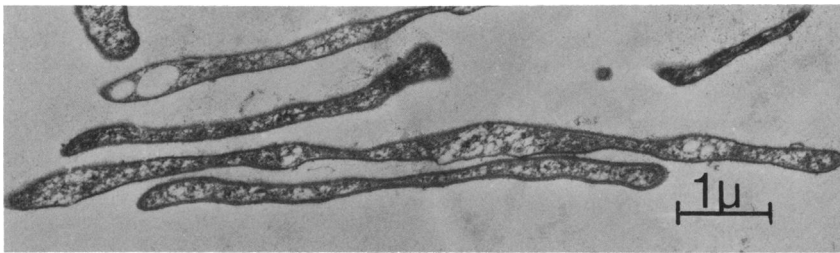


FIG. 8. Cross-sectioned cells at a magnification comparable to that of the scanning electron micrographs, demonstrating the much more irregular shape of the fixed and embedded cells. Magnification,  $\times 13,000$ .

sufficient. The presence in the cell suspensions of bacteriorhodopsin or a closely related pigment in low concentration may also be used as an argument, because this pigment has so far only been found in extreme halophiles. However, pure cultures must be obtained before firm conclusions can be reached.

The observed cell wall structure also presents some problems. Walsby mentioned that he observed a hexagonal lattice of 2-nm particles in shadowed cells. This is a typographical error and should read 20 nm (A. E. Walsby, personal communication). His results, therefore, agree with ours. However, it is generally thought that in halobacteria, the cell wall determines the cell shape, and it is difficult to reconcile the hexagonal lattice with the rectangular shapes of the cells. The difference in the shapes of the rounded cell profiles seen in section and the box-like appearance of the cells in the scanning micro-

graphs also requires an explanation. Although we are probably dealing with a heterogeneous population, which may explain the differences in wall structure we observed, this heterogeneity cannot explain the differences between the scanning and transmission electron micrographs. Virtually all cells had flat edges in the scanning micrographs and rounded edges in sectioned and freeze-fracture preparations. Since it is unlikely that distortions introduced by the technique produced straight and flat edges, we believe that the rounded appearance is the artifact.

We have recently examined brines from salt ponds near Guerrero Negro and La Paz in Baja California, Mexico. In both cases, cells with the same light microscopic appearance as the square species from the Sinai were present in relatively large numbers. Samples from the salt ponds of San Francisco Bay contained about 10 times the total number of cells found in the Sinai and Baja

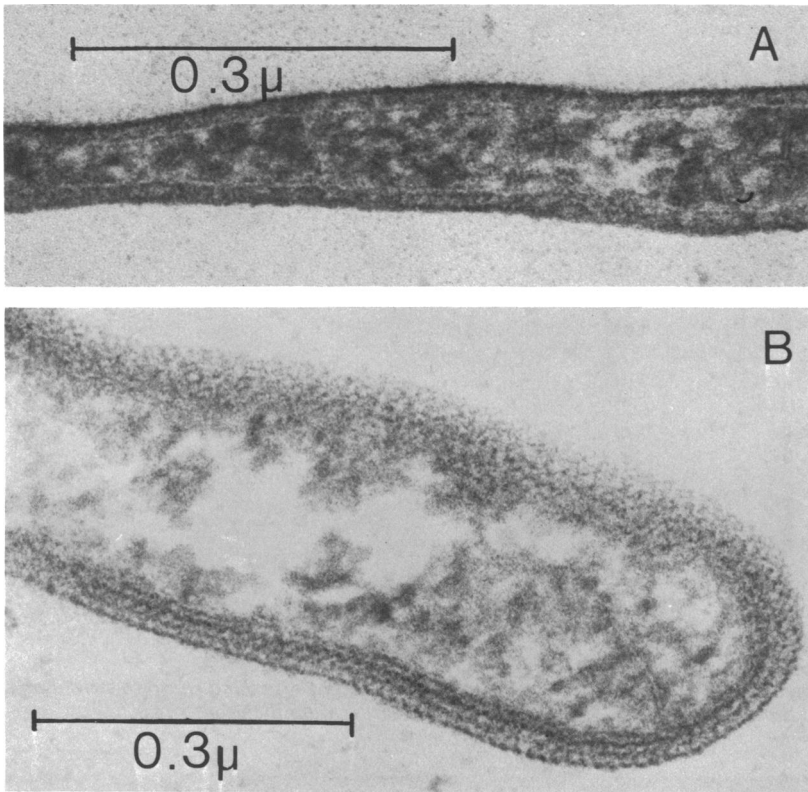


FIG. 9. Cross-section of a cell showing the thick dense wall and the underlying cell membrane, which appears only as a narrow light band (9A). In an oblique section, the regular structure of the wall becomes obvious (9B). Magnifications,  $\times 117,000$  (9A) and  $\times 66,000$  (9B).

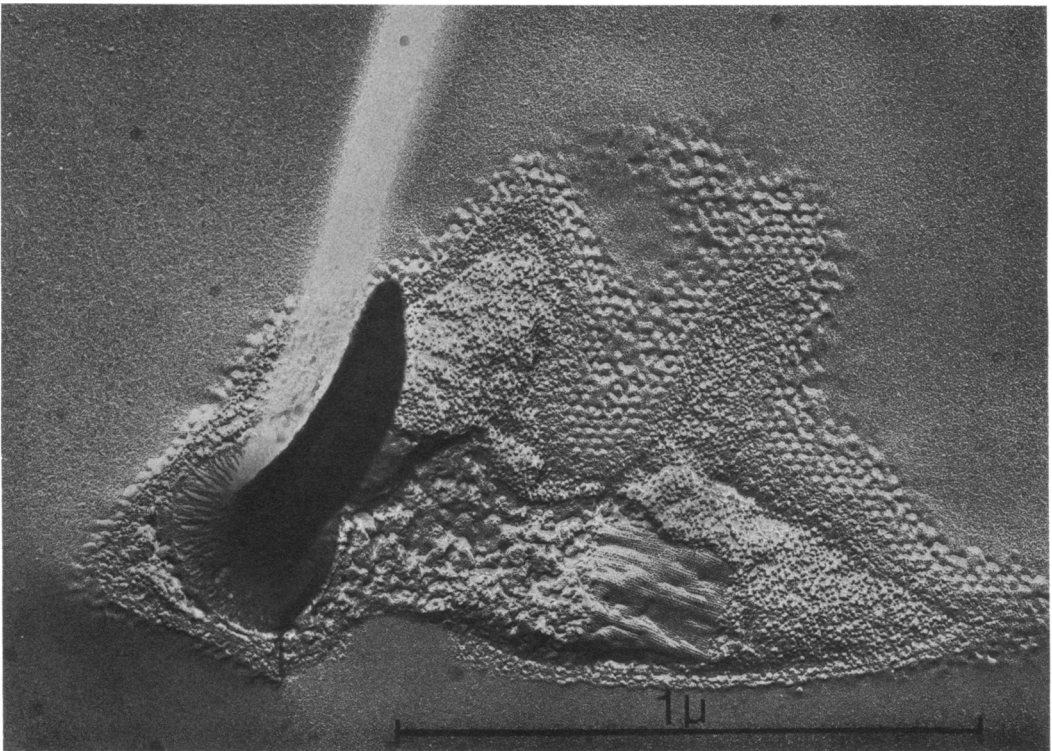


FIG. 10. Freeze-etch electron micrograph. On the upper right hand, the globular structure of the cell wall is exposed by etching. Below it, the cytoplasmic fracture face of the cell membrane shows a much finer and irregular particle distribution. To the left, a granule has been drawn out into a horn-like structure by the fracturing. To the right of it, the cytoplasm has been exposed, and embedded in it is a collapsed gas vacuole with its characteristic fine striation. Magnification,  $\times 76,800$ .

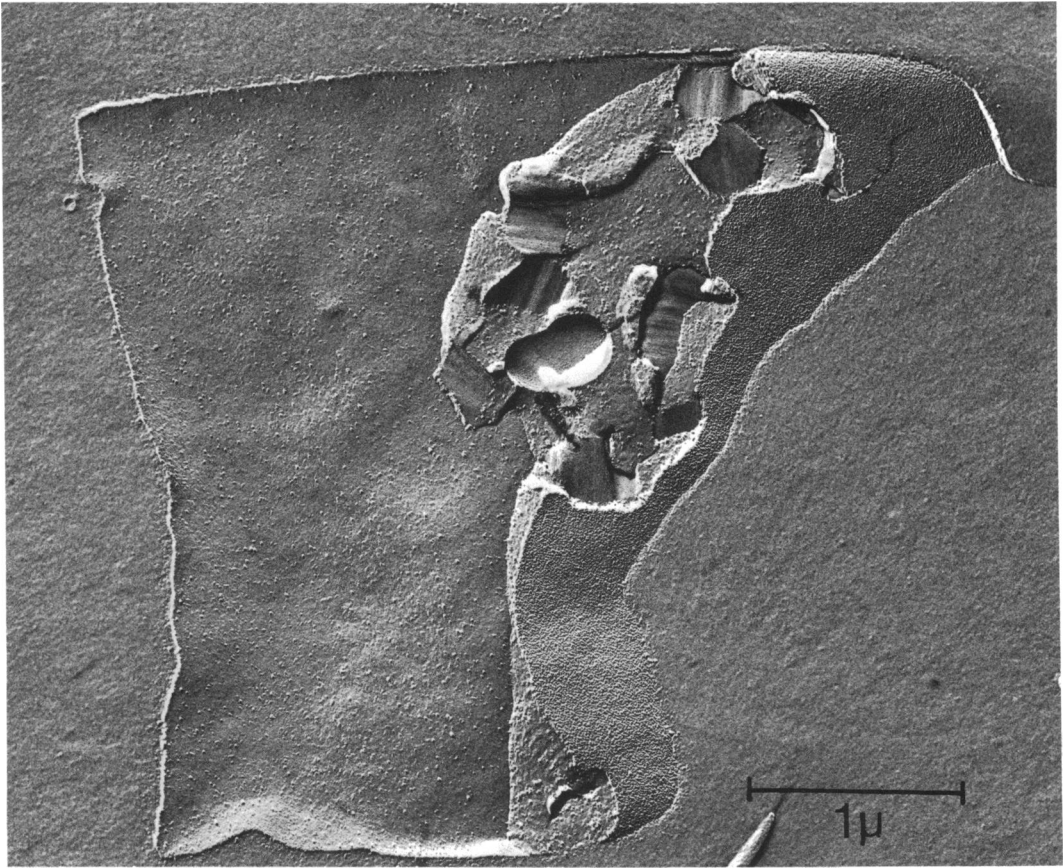


FIG. 11. Freeze-fracture exposing, on the left, the external fracture face of the cell membrane. Next to it is the cytoplasm with collapsed gas vacuoles and a smooth depression left by a granule removed by the fracturing. To the right is the cytoplasmic fracture face of the cell membrane. Magnification,  $\times 28,200$ .

California samples, but square cells were extremely rare. It is, therefore, likely that they can be found in other locations too and, so far, have not been recognized as cells because of their unusual shapes. Occasional particles with a similarly flat but triangular shape have been seen. These are also mentioned by Walsby, but whether they are bacteria related to the square species remains to be seen.

Since cells with this distinct morphology are found in widely separated locations and more related species are likely to be found, the need for a proper generic name arises; "square" bacteria is obviously unsatisfactory. Traditionally, procaryotes have been named according to their shape, and since the shape of these cells in the light microscope and the scanning electron microscope resembles that of a flat box, we tentatively suggest *Arcula* (F. L. n. *arcula*, a small box).

#### ACKNOWLEDGMENTS

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